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Characterization of Three Novel Monoclonal Antibodies Against Hepatitis C Virus Core Protein

Darius Moradpour, Takaji Wakita, Katsutoshi Tokushige, Rolf I. Carlson, Krzysztof Krawczynski, and Jack R. Wands

Molecular Hepatology Laboratory, Massachusetts General Hospital Cancer Center and Department of Medicine, Harvard Medical School, Boston, Massachusetts (D.M., T.W., K.T., R.I.C., J.R.W.); Hepatitis Branch, Centers for Disease Control and Prevention, Atlanta, Georgia (K.K.)

Three novel monoclonal antibodies (MAbs) were established against a recombinant hepatitis C virus (HCV) core protein derived from cloned genotype 1b HCV cDNA. MAbs C7-50 and C8-59 recognize a conserved linear epitope represented by amino acid residues 21 to 40 of the nucleocapsid protein. MAb C8-48 is directed against a strain-specific conformational epitope located within the first 82 amino acids. A sensitive two-site MAb-based immunoradiometric assay was established using antibodies directed against distinct epitopes on the nucleocapsid protein. Processed 21 kDa core protein was detected by immunoblotting in human hepatocellular carcinoma cell lines and primary adult rat hepatocytes transfected with a cytomegalovirus promoter-driven expression construct. Immunofluorescence microscopy studies revealed a granular and vesicular cytoplasmic staining pattern. MAb C7-50 was used successfully to detect HCV core antigen in chronically infected chimpanzee liver tissue. These MAbs represent important reagents for the study of HCV biology and for the development of immunodiagnostic assays.

KEY WORDS: recombinant protein, epitope mapping, immunostaining

INTRODUCTION

Hepatitis C virus (HCV) is the major etiologic agent of posttransfusion and sporadic non-A, non-B hepatitis [Choo et al., 1989; Kuo et al., 1989]. The majority of acutely HCV-infected individuals subsequently develop chronic infection and liver cirrhosis as well as hepatocellular carcinoma (HCC), all well recognized late complications of chronic hepatitis C [Kiyosawa et al., 1990]. As found in flaviviruses and pestiviruses, the single-stranded positive-sense RNA genome codes for a large polyprotein precursor that is posttranslationally processed by cellular and viral proteases to yield mature structural and nonstructural proteins [Choo et al., 1991; Houghton et al., 1991]. Diagnosis of HCV infection is based primarily on the detection of antibodies directed against recombinant viral proteins or on detection of viral RNA by reverse transcription and polymerase chain reaction (RT-PCR). Development of assays that quantitatively detect circulating HCV antigens would be desirable since viral proteins are more stable than RNA, particularly in stored serum samples.

The 21 kDa core protein comprises a basic, RNA-binding aminoterminal domain and a highly hydrophobic carboxyterminal region and is cleaved from the polyprotein precursor by cellular signal peptidase [Hijikata et al., 1991; Santolini et al., 1994]. The nucleocapsid protein is well conserved among different HCV genotypes and may represent an important target antigen for diagnostic assays [Bukh et al., 1994]. Previous studies have shown that the majority of HCV-infected individuals develop antibodies to the HCV core protein early in the course of infection [Chiba et al., 1991; Hosein et al., 1991]. Interestingly, recent observations indicate that the HCV core protein may suppress hepatitis B virus replication in a phosphorylation-dependent manner, suggesting that it may have certain gene regulatory functions as well [Shih et al., 1993, 1995].

The purpose of this study was to develop monoclonal antibodies (MAbs) against the core protein as reagents to study antigenic determinants, the cellular processing, and the localization of the viral nucleocapsid protein in transfected and naturally infected cells. Moreover, as a first step towards the implementation of a quantitative antigen detection assay, a series of two-site MAb-based immunoradiometric assays (mIRMAs) was developed.

MATERIALS AND METHODS

Production of Recombinant HCV Core Protein

HCV cDNAs containing the 5′ noncoding region, the core, and part of the E1 region (nucleotide [nt] 1 to 321)

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Address reprint requests to Jack R. Wands, M.D., Molecular Hepatology Laboratory, Massachusetts General Hospital Cancer Center, 149 13th Street, Charlestown, MA 02129.

were cloned from sera of three individuals with chronic posttransfusion hepatitis C by RT-PCR as described [Wakita and Wands, 1994]. Phylogenetic analysis indicated that cDNA clones CC and CH belonged to genotype 1b and clone CT to genotype 2a (Mizokami M and Ohba K, personal communication). The CC HCV core cDNA (nt 342 to 914) was subcloned into the BamHI-EcoRI sites of a pGEX vector (Pharmacia, Piscataway, NJ) to allow expression of HCV core as a fusion protein with glutathione-S-transferase (GST). HCV core–GST fusion protein was produced in *E. coli* strain DH5α and affinity-purified on glutathione-sepharose beads as described [Iwata et al., 1995]. The production of recombinant full-length and truncated HCV core proteins without GST moiety will be reported elsewhere (Tokushige et al., manuscript in preparation). For in vitro translation, HCV core cDNAs were subcloned into the BamHI-EcoRI sites of pGEM-11Zf(+) and transcribed and translated using the TNT SP6 coupled rabbit reticulocyte lysate system (Promega, Madison, WI).

Establishment of Monoclonal Antibodies to HCV Core Protein

BALB/c mice were immunized with purified HCV core-GST fusion protein, spleen cells were fused with the Sp2/O-Ag14 myeloma cell line (American Type Culture Collection, Rockville, MD), and hybridomas were selected and maintained as previously described [Wands and Zurawski, 1981]. Supernatants were screened by radioimmunoassay, essentially as described [Harlow and Lane, 1988] and hybridomas immunoreactive with recombinant HCV core proteins were cloned by limiting dilution. The MAb isotype was determined with a mouse MAb isotyping kit (Amersham, Arlington Heights, IL). A protein G column was used for affinity purification of MAbs from ascites fluid (Pharmacia, Piscataway, NJ). MAbs were radiolabeled with ^{125}I using the Iodogen method [Harlow and Lane, 1988].

Immunoblotting

Western blot analysis was performed according to a standard protocol [Sambrook et al., 1989]. Horseradish peroxidase-labeled sheep anti-mouse immunoglobulin was used for detection of bound primary antibody by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Competitive Inhibition Assays

For competitive inhibition assays, 96-well polystyrene plates coated with recombinant HCV core protein were simultaneously incubated with 10^5 cpm of radiolabeled MAb per well and increasing amounts (0 to 1.3 μg, corresponding to a 0- to 100-fold molar excess) of unlabeled competing antibody for 1 hour at 20°C. Subsequently, wells were washed with phosphate-buffered saline (PBS), cut out, and counted in a gamma well counter.

Epitope Mapping

A series of synthetic 20-mer peptides covering the entire HCV core region [Iwata et al., 1995] was used for binding and inhibition assays. For binding assays, 96-well polystyrene plates were coated with peptides and incubated with 10^5 cpm of radiolabeled MAb per well. For inhibition experiments, wells were coated with HCV core-GST fusion protein followed by simultaneous incubation with 10^5 cpm of radiolabeled antibody and 10 μg of peptide.

Development of Two-Site mIRMAs

To define the characteristics of the MAbs as potential capture and detection antibodies, 1/4-in polystyrene beads (Precision Plastic Ball Co., Chicago, IL) were coated with 1 μg MAb per bead and then incubated with recombinant HCV core protein and 10^5 cpm of radiolabeled detection MAb for 4 hours at 45°C. Beads were washed four times using the Pentawash system (Abbott, North Chicago, IL) and counted in a gamma well counter.

Eukaryotic HCV Core Expression Constructs

Plasmid pBKCMVΔlacPSTP was derived from the cytomegalovirus promoter-based vector pBKCMV (Stratagene, La Jolla, CA) by removal of the lac promoter between the NheI and SalI sites and insertion of a stop codon linker into the ApaI-KpnI sites. The CH HCV cDNA comprising nt 1 to 1321 was subcloned into the Bam-HI-XbaI sites of pBKCMVΔlacPSTP to yield plasmid pBKCMVCH. To define the epitope recognized by MAb C8-48, nt 333 to 1321 of the CH and CC cDNAs were subcloned into the HindIII-XbaI sites of pcDNA3 (Invitrogen, San Diego, CA) to yield plasmids pCMVHXCH and pCMVHXCC. The HindIII-KpnI fragments (nt 333 to 580) encoding the first 82 amino acid residues of the core protein were exchanged between the two plasmids to yield the chimeric constructs pCMVHKCCKXCH and pCMVHKCHKXCC.

Transfection Experiments

FOCUS [He et al., 1984], HuH-7 [Nakabayashi et al., 1982], and Hep G2 cells [Aden et al., 1979] were maintained in Eagle's Minimal Essential Medium (Mediatech, Washington, DC) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), nonessential amino acids (Gibco BRL, Grand Island, NY), 50 units/ml penicillin G and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 7% CO_2. Primary adult rat hepatocytes (PARH) were prepared by a two-step perfusion technique as previously described [Levitsky et al., 1994]. Eighty percent confluent cells were transfected using a modified calcium phosphate precipitation method [Chen and Okayama, 1987]. For Western blot analysis, transfected cells were lysed in a buffer containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 1% SDS, 50 mM Tris-Cl pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A, followed by shearing of genomic DNA through a 27-gauge needle.

TABLE I. Summary of MAb Characteristics

	C7–50	C8–48	C8-59
Isotype	IgG_1 κ	IgG_1 κ	IgG_1 κ
ELISA	+	+	+
Western blot	+	((+))	+
Indirect immunofluorescence	+	+	+
Radioimmunoassay			
Antigen capture	+	+	+
Antigen detection	+	+	+
Epitope	aa 21–40 linear conserved	aa 1–82 conformational strain-specific	aa 21–40 linear conserved
Immunostaining of HCV-infected liver tissue	+	–	ND

aa, amino acid residue; ND, not done; ((+)), weak reactivity.

Indirect Immunofluorescence Microscopy

Cells grown on microscope cover slides were transfected as described above. Forty-eight hours later, cell monolayers were washed with PBS, fixed in 2% paraformaldehyde, permeabilized with 0.05% saponin, and incubated with 1 μg MAb in 100 μl PBS containing 3% bovine serum albumin and 0.05% saponin. Bound MAb was revealed with a fluorescein isothiocyanate (FITC)–conjugated goat F(ab') fragment to mouse IgG F $(ab')_2$ (Cappel, Durham, NC). Cover slides were mounted in SlowFad (Molecular Probes, Eugene, OR) and examined with a Nikon Labophot photomicroscope equipped with the epifluorescence attachment EF-D (Nikon, Garden City, NY).

Immunostaining of HCV-Infected Liver Tissue

Cryostat sections of liver specimens obtained from chimpanzees during the acute or chronic phase of HCV infection were studied. The animal with acute HCV infection was inoculated with the CDC/Chiron US-1 strain [Bradley et al., 1990]. Chronic HCV infection in the second chimpanzee resulted from multiple inoculations with less well-defined inocula during malaria experiments carried out in the late 1970s. In the specimen obtained during the acute phase of the infection 50 to 70% of hepatocytes contained HCV antigen when stained with purified FITC-labeled IgG fractions from sera of individuals with chronic HCV infection [Krawczynski et al., 1992]. Using the same antibody preparation, several focally distributed hepatocytes were found to contain very large amounts of HCV antigen in sections from the chronically infected chimpanzee. Liver samples used as negative controls included preinoculation specimens and specimens from chimpanzees infected with hepatitis A or B virus. The details of experimental protocols involving these animals and the facilities in which they were housed were approved by the Centers for Disease Control and Prevention institutional research committee. MAbs C7-50 and C8-48 were conjugated with FITC Isomer I (Molecular Probes, Eugene, OR) following a protocol provided by the manufacturer and absorbed with mouse liver powder as described before [Krawczynski et al., 1992]. Cryostat sections were fixed in chloroform for 5 minutes, air dried, and stained with FITC-labeled C7-50 or C8-48 MAb.

RESULTS

Characteristics of Anti-HCV Core MAbs

Three hybridomas producing the HCV core-specific MAbs C7-50, C8-48, and C8-59 were obtained from two separate cell fusions. Table I summarizes the characteristics of these antibodies. Titration curves against recombinant HCV core proteins in an ELISA format indicated that they have comparable affinities for their epitope on the viral nucleocapsid protein. All three MAbs reacted positively down to a concentration of 5 ng/ml, corresponding to a titer of about $1:2 \times 10^8$ in terms of ascites fluid (data not illustrated). C7-50 and C8-59 function well in a Western blot format whereas MAb C8-48 reacts only weakly under these experimental conditions (data not illustrated).

Competitive Inhibition Assays

Figure 1 shows the results of competitive inhibition experiments in which unlabeled MAbs compete with radiolabeled C7-50 for binding to recombinant HCV core protein. Interestingly, increasing amounts of unlabeled C8-59 led to a dose-dependent decrease in binding of labeled C7-50 MAb with complete inhibition observed at a 100-fold molar excess. On the other hand, no inhibition was observed with MAb C8-48. These results indicate that MAbs C7-50 and C8-59 recognize the same or two closely related epitopes whereas C8-48 is directed against a distinct and separate epitope on the HCV core protein.

Epitope Mapping

From the results shown in Figure 2 it is apparent that both C7-50 and C8-59 recognize an epitope defined by peptide CP-3 which represents amino acid residues 21 to 40 of the HCV core protein. The epitope recognized by MAb C8-48 could not be determined by this approach. In another set of experiments, however, C8-48 was found by Western blot analysis to react weakly with the CC cDNA-derived recombinant protein which was used to raise the antibody, but no reactivity was found with

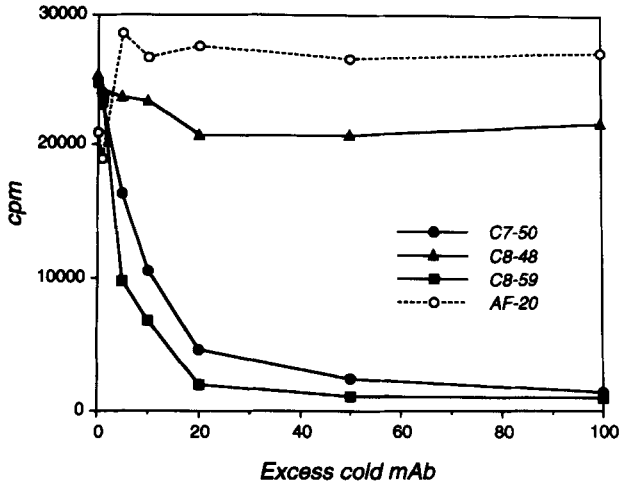

Fig. 1. Competitive inhibition assay. Wells coated with HCV core-GST fusion protein were incubated simultaneously with radiolabeled MAb C7-50 and a 0- to 100-fold molar excess of the indicated unlabeled antibodies as described in Materials and Methods. AF-20 is a nonrelevant control MAb [Wilson et al., 1988]. Mean values of duplicate determinations are shown.

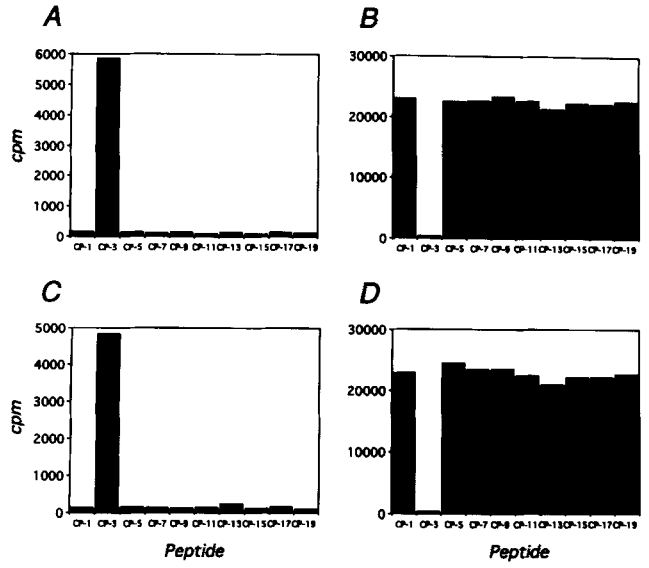

Fig. 2. Epitope mapping of HCV core antigenic determinants. Binding (**A** and **C**) and inhibition (**B** and **D**) assays were performed with a series of synthetic 20-mer peptides representing the entire core protein as described in Materials and Methods. Mean values of duplicate determinations obtained from MAbs C7-50 (A and B) and C8-59 (C and D) are shown. Peptide CP-3 represents amino acid residues 21 to 40 of the HCV core protein.

proteins derived from the CH and CT clones (data not illustrated). In addition, we found that C8-48 reacted by immunofluorescence staining only with cells transfected with constructs expressing the CC but not the CH HCV core cDNA clone. The epitope recognized by MAb C8-48 could therefore be mapped by cassette exchange experiments. Indeed, C8-48 stained only cells transfected with pCMVHXCC and pCMVHKCCKXCH, but not pCMVHXCH and pCMVHKCHKXCC, whereas C7-50 reacted with cells transfected with all four constructs (data not illustrated). These experiments indicate that MAb C8-48 recognizes a strain-specific epitope defined by the first 82 amino acids of the HCV core protein. Moreover, the discrepancy between strong binding to HCV core protein in an ELISA format and by immunostaining, in which antigens are presented in native conditions, and only weak reactivity in a Western blot format suggests that C8-48 recognizes a conformational epitope whereas C7-50 and C8-59 are directed against linear epitopes.

Two-Site mIRMAs

The results of a series of two-site mIRMAs for the detection of HCV core protein are summarized in Figure 3. Various combinations of capture and radiolabeled (indicated by asterisks) detection MAbs were examined. A sensitive assay was established by the combination of two MAbs that recognize distinct epitopes on the HCV core protein (C8-48 and *C7-50 or *C8-59 or vice versa). The detection threshold of these assays is in the range of a few ng/ml. The observation that a functional mIRMA may be obtained using the same MAb for antigen capture and detection (e.g., C7-50 and *C7-50) suggests that recombinant core protein may aggregate or assemble into dimers or multimeric particles.

Detection of Processed HCV Core Protein in Transfected HCC Cell Lines and PARH

As shown in Figure 4, processed 21 kDa core protein was detected by C7-50 MAb in HuH-7, Hep G2, and FOCUS cells and in PARH transfected with pBKC-MVCH.

Indirect Immunofluorescence Microscopy

Using MAb C7-50, these experiments revealed a granular cytoplasmic staining pattern with a predominant perinuclear distribution (Fig. 5). In addition, conspicuous vesicular structures of various amounts and sizes were identified. Similar results were obtained with MAbs C8-48 and C8-59 and in transfected Hep G2 and FOCUS cells (data not illustrated).

Immunostaining of HCV-Infected Liver Tissue

MAb C7-50 did not show any reactivity with hepatocytes containing small amounts of HCV antigen detectable by polyclonal human antibodies in the chimpanzee liver specimen from the acute phase of HCV infection. However, when reacted with the chronic phase specimen known to contain massive deposits of HCV antigen, C7-50 MAb reacted with single cells. The cytoplasmic deposits were mostly homogenous and rather weak with superimposed more intensely stained granules (Fig. 6A and B). Very rarely, single cells contained brilliant HCV core-positive granules in the cytoplasm. A group of stronger reactive cells was found in a single section from this liver specimen (Fig. 6C). Some of the positive cells in this group reacted strongly with the MAb and some had weaker fluorescence, indicating that the amount of HCV

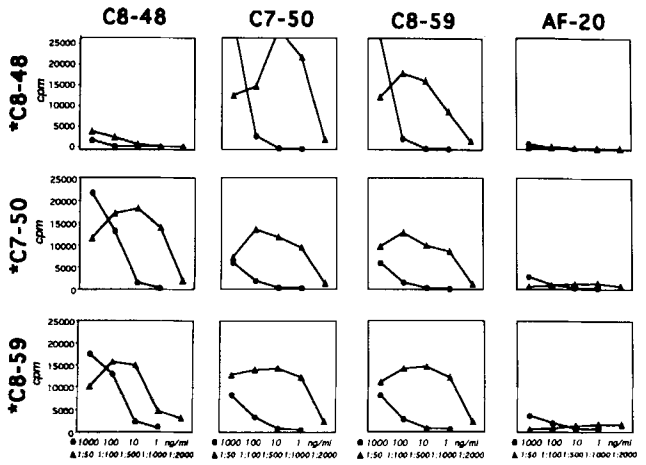

Fig. 3. Two-site mIRMAs for the detection of HCV core protein. Decreasing concentrations of purified HCV core-GST fusion protein (circles) and serial dilutions of a crude bacterial lysate containing full-length recombinant HCV core protein without GST moiety (triangles) were assayed in a series of two-site mIRMAs with different unlabeled capture antibodies (indicated on top) and radiolabeled detection antibodies (indicated on the left and denoted by asterisks) as described in Materials and Methods. Mean values of duplicate determinations are shown. AF-20 is a nonrelevant control MAb.

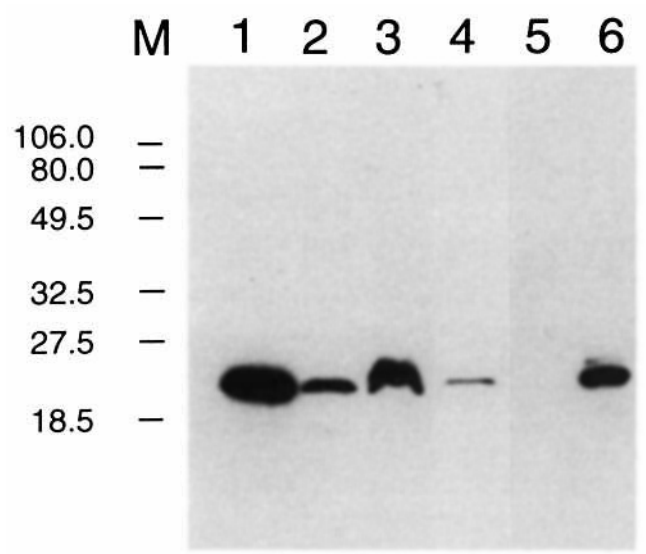

Fig. 4. Analysis of HCV core gene expression in transfected human HCC cell lines and in PARH. HuH-7 **(lane 1)**, Hep G2 **(lane 2)**, FOCUS **(lane 3)** cells, and PARH **(lane 4)** were transfected with pBKCMVCH. Cell lysates were prepared 48 hours after transfection and analyzed by 15% SDS-PAGE and immunoblotting using MAb C7-50 as described in Materials and Methods. HuH-7 cells mock-transfected with pBKCMVΔlacPSTP **(lane 5)** and in vitro translated HCV core protein **(lane 6)** served as negative and positive controls, respectively. Molecular weight standards in kDa are shown on the left.

core antigen varied in the positive hepatocytes. No nuclear or sinusoidal reactivity was observed. Staining reactions with C8-48 MAb were negative.

DISCUSSION

We have established and characterized three novel MAbs to the HCV core protein and have applied them to investigate properties of the viral nucleocapsid protein. Binding and inhibition studies using a series of synthetic peptides revealed that MAbs C7-50 and C8-59, obtained from two separate hybridoma fusions, recognize the same or two closely related epitopes residing within amino acid residues 21 to 40 of the protein. Good binding to peptides and strong immunoreactivity to HCV core protein in Western blot analysis argue in favor of a linear epitope recognized by these two MAbs. This epitope is highly conserved among all HCV genotypes [Bukh et al., 1994] and was also present on HCV core protein expressed in transfected cells and in HCV-infected liver tissue. Moreover, it coincides with an immunodominant epitope previously identified in HCV-infected individuals. Nasoff et al. [1991], for example, found that 84% of sera derived from HCV-infected individuals recognize a peptide spanning amino acids 21 to 40. Furthermore, a linear epitope recognized by the human MAb (hMAb) U1/F10 was mapped to amino acid positions 34 to 39 [Siemoneit et al., 1994]. Finally, with regard to the mapping of the epitope recognized by MAb C8-48, our study in the murine system as well as studies in humans [Nasoff et al., 1991; Cerino et al., 1993; Goeser et al., 1994] suggest that there is at least one additional major conformational epitope residing within the first 82 amino acids of the protein. To directly compare the epitopes recognized by the mouse with those recognized by humans, we performed competitive inhibition experiments with hMAb B12.F8 which recognizes a conformational epitope centered around amino acids 34 to 45 [Cerino et al., 1993] and hMAb U1/F10 [Siemoneit et al., 1994] and found that both bound with high affinity to our recombinant proteins but that neither competed for binding with MAb C7-50 or C8-48 (data not illustrated). These results suggest that the immunodominant amino-terminal region of the HCV nucleocapsid protein contains multiple closely situated major antigenic determinants. These observations are reminiscent of the multiple epitopes found on the major antigenic *a* determinant of the hepatitis B virus surface antigen [Wands et al., 1982].

Cassette exchange and transfection experiments revealed that MAb C8-48 recognizes a strain-specific epitope encoded by the CC but not by the CH cDNA. This is remarkable because both cDNAs were derived from individuals infected with HCV strains of genotype 1b and indicates that minor variations in the generally well-conserved core protein may abrogate important antigenic determinants. Such mechanisms may contribute to viral persistence during chronic HCV infection.

The results of two-site mIRMAs suggest that these antibodies may be valuable tools for the development of a core antigen assay. However, given the fact that HCV circulates at low titers during acute and chronic infection, these assays are not sensitive enough, in their present format, to detect HCV core antigen in serum. Indeed, using a murine MAb as capture antibody and affinity-purified human antibodies as detection antibodies in an ELISA with a similar sensitivity, Takahashi et al. [1992] were able to detect HCV core protein only in a virus-

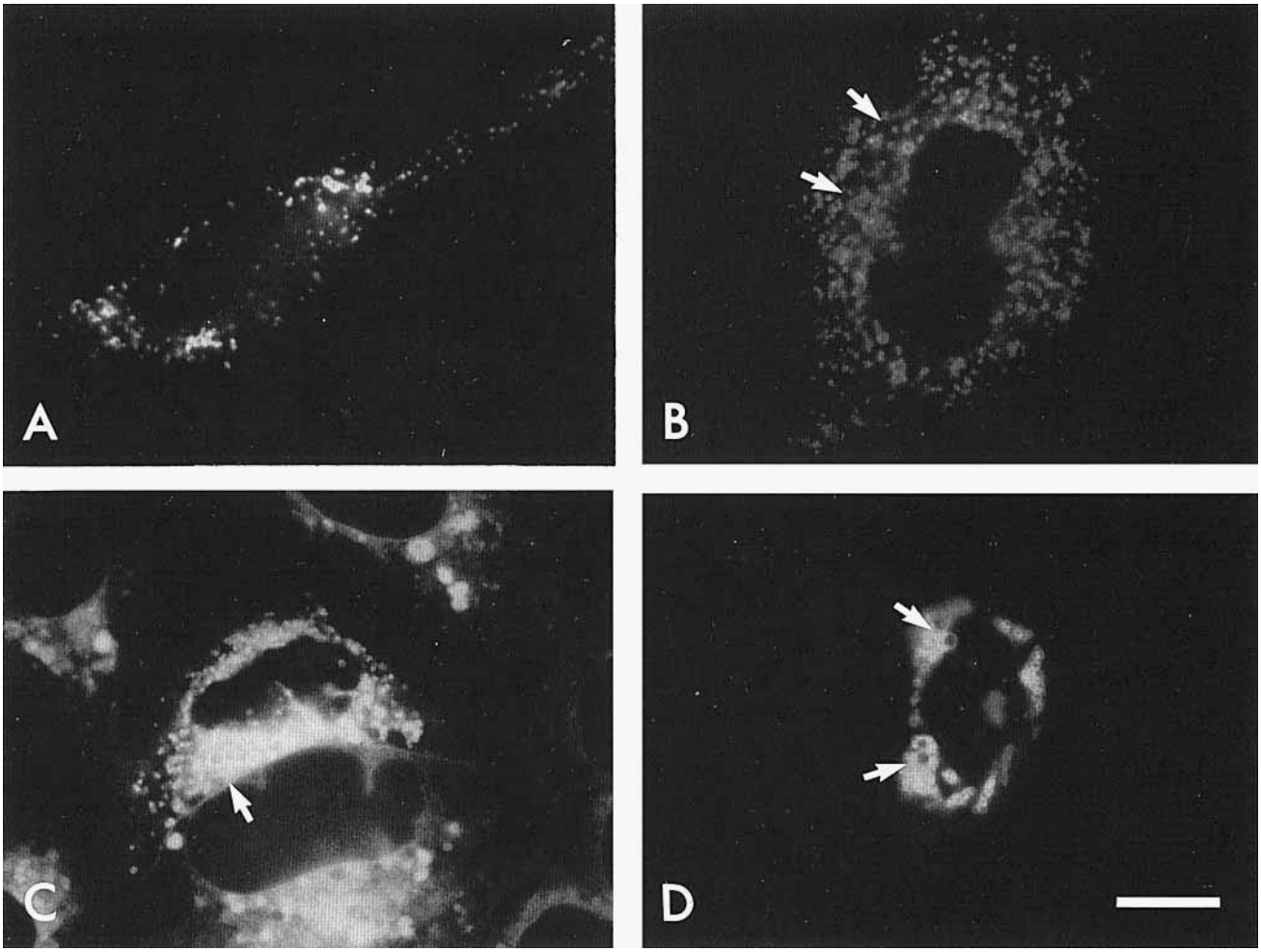

Fig. 5. Indirect immunofluorescence staining of pBKCMVCH-transfected HuH-7 cells. HuH-7 cells grown on microscope cover slides were transfected with pBKCMVCH and processed 48 hours later for detection of HCV core protein by indirect immunofluorescence microscopy using MAb C7-50 as described in Materials and Methods. **A** and **B** show a typical granular cytoplasmic staining pattern. A vesicular staining pattern is apparent in B, **C,** and very prominent in **D** (arrows). C was counterstained with Evans blue to demonstrate the presence of nontransfected cells surrounding the positive cell. Original magnification 1,440×. Calibration bar represents 10 μ.

enriched, detergent-treated plasma fraction derived from HCV-infected individuals. To overcome the need for concentration of serum samples we are currently developing an immuno-PCR-based assay to amplify the signal obtained from a two-site mIRMA. Previous studies with hepatitis B virus surface antigen suggest that antigen detection may be improved 200- to 400-fold by this approach [Maia et al., 1995].

Consistent with earlier reports [Harada et al., 1991; Grakoui et al., 1993; Selby et al., 1993; Santolini et al., 1994], expression of an subgenomic HCV cDNA fragment containing the core and part of the E1 region led to the production of a 21 kDa product believed to represent the processed HCV nucleocapsid protein in the human HCC cell lines HuH-7, Hep G2, and FOCUS, and in PARH. The demonstration of processed HCV core protein in primary hepatocytes suggests that the 21 kDa protein indeed represents a genuine product of the polyprotein processing in the liver.

Similar to our studies, Santolini et al. [1994] observed by immunofluorescence microscopy small round structures in the cytoplasm of core-transfected CV-1 cells. In the present investigation, vesicular structures of varying amount and size were found together with a granular cytoplasmic staining pattern. These vesicles gave a ring-like fluorescence pattern and contained an empty inner space, suggesting that core protein was associated with the vesicle membrane. Interestingly, alteration of intracellular membranes with vesicle formation was found to be a prominent feature of flavivirus infection [Chambers et al., 1990]. However, additional studies are needed to address the issue of whether the vesicular staining pattern observed in our expression system represents a cellular compartment naturally implicated in HCV assembly or an artifact of core protein overexpression. In contrast to the findings of Shih et al. [1993], we were unable to demonstrate a nuclear localization of full-length HCV core protein.

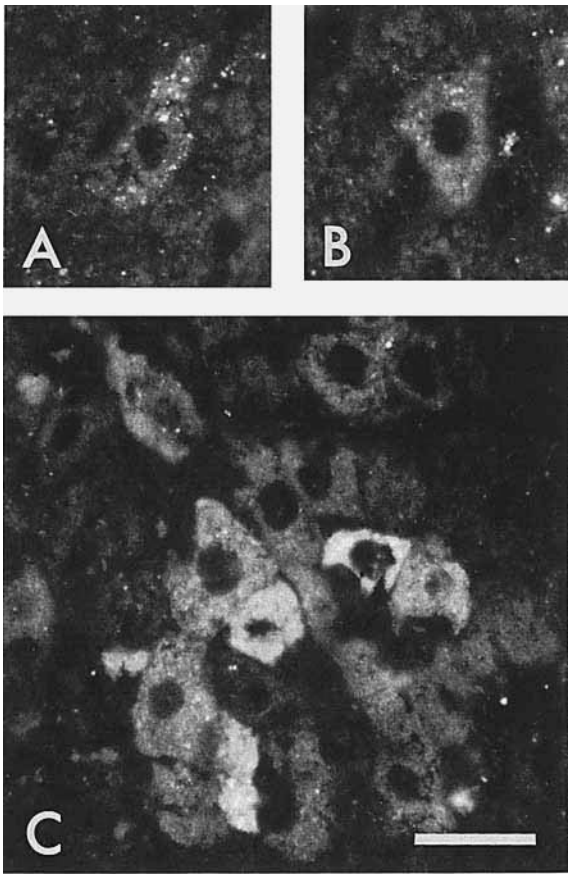

Fig. 6. Direct immunofluorescence staining of chronically HCV-infected chimpanzee liver tissue. MAb C7-50 was used as described in Materials and Methods. **A** and **B** show single hepatocytes containing HCV core antigen in the cytoplasm. Note stronger granular fluorescence superimposed on weaker homogenous cytoplasmic deposits. White granules outside hepatocytes are due to autofluorescence of iron deposits (yellowish in the original illumination, in contrast to the apple-green fluorescence of FITC). **C** shows a group of hepatocytes with HCV core antigen. Original magnification 504×. Calibration bar represents 20 μ.

Immunostaining of liver tissue of a chronically HCV-infected chimpanzee confirmed that MAb C7-50 recognizes a naturally occurring epitope on the HCV core protein. This antibody reacted only with hepatocytes from the chronically infected chimpanzee which had very high levels of HCV antigen as judged by immunostaining with polyclonal human antibodies. We believe, therefore, that the failure to detect core antigen in liver specimens obtained from the acutely infected animal represents a quantitative rather than a qualitative phenomenon. MAb C8-48 did not react with infected liver tissue likely because of its strain-specificity. Further studies using liver samples from individuals with high levels of HCV, for example from patients with recurrent HCV infection after liver transplantation, will be necessary to gain more information about the pattern and clinicopathological significance of HCV core antigen detection in liver tissue.

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